



NCBI Primer-BLAST

An online tool for designing target-specific PCR primer pairs (with internal probes)

<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>

National Center for Biotechnology Information • National Library of Medicine • National Institutes of Health • Department of Health and Human Services

Scope and Access

Primer-BLAST [1] is a PCR primer design and specificity checking tool from NCBI. It first scans the database to identify unique regions in the supplied template, picks primers from these regions using the Primer3 algorithm [2], and screens the returned primer sets using BLAST [3] to evaluate their specificity to the input template. It presents candidate primers along with their alignment to identified target sequences in the database. Primer-BLAST is a web only application accessible through the “Specialized BLAST” section of the BLAST homepage (blast.ncbi.nlm.nih.gov/) or directly through the Primer-BLAST search form at www.ncbi.nlm.nih.gov/tools/primer-blast/.



Accepted Inputs

The Primer-BLAST search page (right & below) contains multiple sections. The “PCR Template” (A) takes your input template, for which you can limit where the forward and reverse primers has to be picked up from (B). With a valid template, Primer-BLAST will find a set of primer pairs optimal for PCR amplification. The “Primer Parameter” accepts different combinations of primer input (C), such as a primer pair with its template, a template with a single primer, and a pair of primers alone. With specificity check option selected (default), Primer-BLAST will screen primer pairs for their specificities and report any potential off-target annealing. With a single primer input, Primer-BLAST finds candidate primers working with the input template and primer. For primer pairs only input, Primer-BLAST finds the amplification target in selected organism and return the primer template alignments. With a RefSeq mRNA accession as an input template, Primer-BLAST can take exon junctions into consideration when finding optimal primer pairs, through the intron inclusion option (D) in the “Exon/intron selection” section. More information on the parameter is available in the popup (E) activated through the question mark click.

The “Primer Specificity Checking Parameters” section (F) provides databases and organism limit selection (G). To increase the chance of finding specific primers, check the splice variant option (H). Change the database to “Custom” (I) option to upload a custom set of sequences (accessions or FASTA) for use as the specificity checking database.

The newly introduced “Primers common for a group of sequences” tab (J) has the same setup, but the template input box takes a group of similar sequences as input and designs PCR primers that amplify all the input templates.

The screenshot displays the NCBI Primer-BLAST web interface. Key sections are labeled with letters A through J:

- A:** PCR Template input field.
- B:** Range selection for forward and reverse primers.
- C:** Primer Parameters section, including PCR product size, number of primers to return, and primer melting temperatures.
- D:** Exon/intron selection options, including intron inclusion and intron length range.
- E:** A popup window providing detailed information about the intron inclusion option.
- F:** Primer Pair Specificity Checking Parameters section.
- G:** Database and organism selection options.
- H:** Allow splice variants checkbox.
- I:** Custom database selection option.
- J:** Primers common for a group of sequences tab.

Additional features include a "Get Primers" button, a "Show results in a new window" checkbox, and a "Use new graphic view" checkbox. A note at the bottom states: "Note: Parameter values that differ from the default are highlighted in yellow."

Advanced Parameters for Primer-BLAST

Clicking the “Advanced Parameters” link (A) toggles on infrequently adjusted parameters. Parameters in the “Primer Pair Specificity Checking Parameters” (B) specify the exhaustiveness of specificity checking and how many results to display. The “Primer Parameters” (C) specify the T_m of the product, and specific properties of the returned primers pairs. In favor of search speed, Primer-BLAST does not use thermodynamic alignment features (D) by default. Settings in buffer condition can greatly affect the primer T_m calculation and you can adjust them here (E). Check the checkbox (F) to instruct Primer-BLAST to take SNPs mapped to template into consideration during primer picking. This only works for human sequence when RefSeq accession is the input template.

The screenshot shows the 'Advanced parameters' section of the Primer-BLAST web interface. Callouts A through J highlight specific features:

- A:** 'Advanced parameters' link.
- B:** 'Primer Pair Specificity Checking Parameters' section, including:
 - Max number of Blast target sequences: 50000
 - Blast expect (E) value: 30000
 - Blast word size: 7
 - Max primer pairs to screen: 500
 - Max targets to show (for designing new primers): 20
 - Max targets to show (for pre-designed primers): 1000
 - Max targets per sequence: 100
- C:** 'Primer Parameters' section, including:
 - PCR Product T_m: Min, Opt, Max fields.
 - Primer Size: Min (15), Opt (20), Max (25).
 - Primer GC content (%): Min (20.0), Max (80.0).
 - GC clamp: 0
 - Max Poly-X: 5
 - Max 3' Stability: 9
 - Max GC in primer 3' end: 5
 - Secondary Structure Alignment Methods:
 - ☐ Use Thermodynamic Oligo Alignment
 - ☐ Use Thermodynamic Template Alignment (warning: search may be slow)
 - TH: Max Template Mispriming: Primer (40.00), Pair (70.00)
 - TH: Max Self Complementarity: Primer (45.0), Pair (35.0)
 - TH: Max Pair Complementarity: Primer (45.0), Pair (35.0)
 - TH: Max Primer Hairpin: 24.0
 - Max Template Mispriming: Primer (12.00), Pair (24.00)
 - Max Self Complementarity: Primer (8.00), Pair (3.00)
 - Max Pair Complementarity: Primer (8.00), Pair (3.00)
 - Excluded regions: text input
 - Overlap junctions: text input
 - 5' side overlaps: 7
 - 3' side overlaps: 4
 - Minimal number of nucleotides that the left or right primer must overlap: 7
 - Concentration of monovalent cations: 50.0
 - Concentration of divalent cations: 1.5
 - Concentration of dNTPs: 0.6
 - Salt correction formula: SantaLucia 1998
 - Table of thermodynamic parameters: SantaLucia 1998
 - Annealing Oligo Concentration: 50.0
 - SNP handling: ☐ Primer binding site may not contain known SNP
 - Repeat filter: Automatic
 - Low complexity filter: ☒ Avoid low complexity region for primer selection
- D:** 'Secondary Structure Alignment Methods' section.
- E:** 'Concentration of monovalent cations', 'Concentration of divalent cations', 'Concentration of dNTPs', and 'Salt correction formula' fields.
- F:** 'SNP handling' checkbox.
- G:** 'Internal hybridization oligo parameters' section, including:
 - Hybridization oligo: ☐ Pick internal hybridization oligo
 - Hyb Oligo Size: Min (18), Opt (20), Max (27)
 - Hyb Oligo tm: Min (57.0), Opt (60.0), Max (63.0)
 - Hyb Oligo GC%: Min (20.0), Opt (50), Max (80.0)
- H:** 'Use new graphic view' checkbox.
- I:** 'Get Primers' button.
- J:** 'Show results in a new window' checkbox.

You can pick internal probe for real-time PCR by activating and adjusting options given in the “internal hybridization oligo parameters” (G). An option of “Use new graphic view” (H), checked by default, allows Primer-BLAST to create a visually informative and interactive summary of the result using the embedded Graphical Sequence Viewer [4].

Submitting a Search

Click the “Get Primers” button (I) to submit the search. The browser tracks the progress of the submitted job via an intermediate polling page (J) and displays the result when it becomes available. You can manually check it by using the “Check” link (K).

Primer-BLAST

A tool for finding specific primers

Making primers specific to your PCR template. [more...](#)

Status	Running	Check	Cancel
Current time	26 December 2020, 16:59:12		
Time since submission	30 sec		
Progress Message			

Primer-BLAST Results: the Graphical Summary

The Primer-BLAST displays results by breaking them into the search summary, the “Graphical view of primer pairs”, and a “Detail primer reports” sections.

The summary section reiterates the template (A) and provides a “Search Summary” link with detailed statistics of the search (B). The informational message (C) provides additional details on the primers returned, with a link to online document with tips.

Primer-BLAST >> JOB ID: ODLnRzPMPmQZWjtfNj8fbUwkDI9hNxVCYA

Search parameters and other details

Number of Blast hits analyzed	338307
Entrez query	
Min total mismatches	2
Min 3' end mismatches	2
Defined 3' end region length	5
Mismatch threshold to ignore targets	6
Max target size	4000
Max number of Blast target sequences	50000
Blast E value	30000
Blast word size	7
Max candidate primer pairs	500
Min PCR product size	70
Max PCR product size	1000
Min Primer size	15
Opt Primer size	20
Max Primer size	25
Min Tm	57
Opt Tm	60
Max Tm	63
Max Tm difference	3
Repeat filter	AUTO
Low complexity filter	Yes

Primer-BLAST Results

Input PCR template (A): NM_000410.4 Homo sapiens homeostatic iron regulator (HFE), transcript variant 1, mRNA

Range: 1 - 5176

Specificity of primers (C): Primers may not be specific to the input PCR template as targets were found in selected database: Refseq mRNA (Organism limited to Homo sapiens)...[help on specific primers](#)

Other reports: [Search Summary](#) (B)

Graphical view of primer pairs (D)

Primer pairs for job (E):

- Primer 1: Forward: 41..60 length 20 Tm 59.17 GC 55.00% Seq TTACTGGGCATCTCTGAGC
- Primer 2: Reverse: 604..623 length 20 Tm 59.96 GC 55.00% Seq AATCCAGTGTGTCAAGGCAG
- PCR product length: 583
- Links & Tools: BLAST nr: NM_000410.3 (41..623), BLAST to Genome: NM_000410.3 (41..623), FASTA record: NM_000410.3 (41..623), GenBank record: NM_000410.3 (41..623)

Configure Page (H):

Tracks: Active Tracks, Search Tracks, Variation, Sequence, Genes/Products, Features, Uploaded Data

Custom Data: Active, Track name

Track Settings: Cited Variations, dbSNP b154 v2 (Track legend): dbSNP 2.0 Build 154 v2 all data based on Homo sapiens

Rendering options: Show variants for 50 or less

Buttons: Configure, Reset tracks, Cancel

Right-click menu (G): Set New Marker At Position, Set Sequence Origin At Position, Flip Sequence Strands, Zoom In, Zoom Out, Zoom To Sequence, Zoom On Range, BLAST and Primer Search, Download, Configure tracks

SNP summary (I): rs774186188, Variation ID: rs774186188, Variation Type: SNV, length 1, Alleles: G/T, [Genomic locations] GCF_000001405.38: NC_000006.12 @ 26091419, GCF_000001405.25: NC_000006.11 @ 26091647, [Links & Tools] SNP summary: rs774186188

With RefSeq accession (NM_000410.3 in this case) as template, the “Graphical view of primer pairs” section provides much more detailed information:

- A clear overview of the results in the context of the target sequence’s annotation, showing the exon boundaries of the template plus its protein product (D)
- Candidate primer pairs with their predicted products (E)
- Properties of a specific primer pair, viewable in the popup (F) upon mouse hovering
- Sequence-level details of the annealing sites through the right-click menu’s “Zoom to Sequence” option (G)
- The relationship of suggested primers with other features, such as SNPs mapped to the template, through the “Configure page” dialog box (H) activated by clicking the “Tracks” button
- Additional feature details, viewable in the popup (I) upon mouse hovering

Primer-BLAST Results: Primer Pairs and Their Alignment to Targets

Detailed primer reports

You can re-search for specific primers by accepting some of the unintended targets, check the box(es) next to the ones you accept and try again to re-search for specific primers. [Submit](#)

Primer pair 1 **A**

	Sequence (5'→3')	Template strand	Length	Start	Stop	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TGATCATGAGAGTCGCCGTG	Plus	20	195	214	59.90	55.00	6.00	1.00
Reverse primer	ACAGCCAAGGTTATCCAGCC	Minus	20	827	808	60.03	55.00	4.00	1.00

Product length 633
Total intron size 1304 (between pos. 8824 and 10405 on [NG_008720.2](#))

Products on intended targets

>NM_000410.4 Homo sapiens homeostatic iron regulator (HFE), transcript variant 1, mRNA

product length = 633

Forward primer	1	TGATCATGAGAGTCGCCGTG	20
Template	195	214
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	827	808

Products on potentially unintended templates **B**

☐ >NM_139006.3 Homo sapiens homeostatic iron regulator (HFE), transcript variant 6, mRNA **C**

product length = 591

Forward primer	1	TGATCATGAGAGTCGCCGTG	20
Template	195	214
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	785	766

☐ >NM_015477.3 Homo sapiens SIN3 transcription regulator family member A (SIN3A), transcript variant 2, mRNA **D**

product length = 3203

Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	3865	...CT.C..C.....A.	3846
Reverse primer	1	ACAGCCAAGGTTATCCAGCC	20
Template	663	.G..T..T.C.C.....	682

The “Detailed Primer Reports” section (left) contains the details for returned primer pairs. Each primer pair is in its own subsection (**A**), which lists basic primer properties along with alignments to their intended target. Alignments to potentially unintended targets (**B**) are at the end of the subsection.

In this example, the first primer pair for human HFE gene transcript variant 1 (NM_000410) also amplifies other variants, such as variant 6 (**C**). Alignments (with mismatches) to a truly unintended target SIN3 gene transcripts are also shown (**D**).

More on “User guided” Mode and “Custom” Database

The search form’s “Primer Pair Specificity Checking Parameters” section provides the “User guided” (**E**) mode to allow Primer-BLAST to distinguish between the intended template and other targets that are highly similar to it (such as other transcript variants from the same gene) upon the job submission (**F**).

Selecting custom database (**G**) allows you to provide custom dataset for specificity checking. System constraints limit the size of sequence files to 300 MB. For sequences already deposited in the NCBI Nucleotide database, you can use their accessions to specify a larger custom dataset.

Primer Pair Specificity Checking Parameters

Specificity check ☒ Enable search for primer pairs specific to the intended PCR template

Search mode **User guided** **E**

Database **User guided**

Organism Enter an organism name (or organism group name such as enterobacteriaceae, rodents), taxonomy id or select from the suggestion list as you type.

NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).

Input PCR template **NM_000249.3** Homo sapiens mutL homolog 1 (MLH1), transcript variant 1, mRNA

Range 1 - 2662

Your PCR template is highly similar to the following sequence(s) from the search database. To increase the chance of finding specific primers, please review the list below and select all sequences (within the given sequence ranges) that are intended or allowed targets.

Select: **All** None Selected: 0

Accession	Title	Identity	Alignment length	Seq. start	Seq. stop
<input checked="" type="checkbox"/> XM_005265164.1	PREDICTED: Homo sapiens mutL homolog 1 (MLH1), transcript variant X3, mRNA	99.8%	2520	1	2515

F

[Submit](#) ☐ Show results in a new window

References

- Ye J, Coulouris G, Zaretskaya I, Cutcutache I, Rozen S, Madden TL. (2012) Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics*. 13:134.
- Rozen, S and Skaletsky, HJ (2000) Primer3 on the WWW for general users and for biologist programmers. In: Krawetz S, Misener S (eds) *Bioinformatics Methods and Protocols: Methods in Molecular Biology*. Humana Press, Totowa, NJ, pp 365-386.
- Altschul, SF, Madden, TL, Schäffer, AA, Zhang, J, Zhang, Z, Miller, W and Lipman, DJ (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." *Nucleic Acids Res.* 25:3389-3402.
- The Graphical Sequence Viewer Factsheet: https://ftp.ncbi.nlm.nih.gov/pub/factsheets/Factsheet_Graphical_SV.pdf.

Primer Pair Specificity Checking Parameters

Specificity check ☒ Enable search for primer pairs specific to the intended PCR template

Search mode **Automatic**

Database **Custom** **G**

Exclusion **Exclude**

Organism **Custom**

Technical Assistance

Please send your feedback, questions and bug reports to blast-help@ncbi.nlm.nih.gov